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Pulmonary infection of mice with human metapneumovirus induces local cytotoxic T-cell and immunoregulatory cytokine responses similar to those seen with human respiratory syncytial virus

Karen A. Herd,[1] Michelle Nelson,[2] Suresh Mahalingam[2]† and Robert W. Tindle[1]†

Correspondence
Robert W. Tindle
r.tindle@uq.edu.au

[1]Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, and Clinical Medical Virology Centre, University of Queensland, Brisbane, Australia

[2]Centre for Biomedical, Molecular and Chemical Sciences, Faculty of Applied Science, University of Canberra, ACT, Australia

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Human metapneumovirus (hMPV) is a major cause of upper and lower respiratory-tract infection in infants, the elderly and immunocompromised individuals. Virus-directed cellular immunity elicited by hMPV infection is poorly understood, in contrast to the phylogenetically and clinically related pathogen human respiratory syncytial virus (hRSV). In a murine model of acute lower respiratory-tract infection with hMPV, we demonstrate the accumulation of gamma interferon (IFN-γ)-producing $CD8^+$ T cells in the airways and lungs at day 7 post-infection (p.i.), associated with cytotoxic T lymphocytes (CTLs) directed to an epitope of the M2-1 protein. This CTL immunity was accompanied by increased pulmonary expression of Th1 cytokines IFN-γ and interleukin (IL)-12 and antiviral cytokines (IFN-β), as well as chemokines Mip-1α, Mip-1β, Mig, IP-10 and CX3CL1. There was also a moderate increase in Th2-type cytokines IL-4 and IL-10 compared with uninfected mice. At 21 days p.i., a strong CTL response could be recalled from the spleen. A similar pattern of CTL induction to the homologous M2-1 CTL epitope of hRSV, and of cytokine/chemokine induction, was observed following infection with hRSV, highlighting similarities in the cellular immune response to the two related pathogens.

INTRODUCTION

Human metapneumovirus (hMPV) was first isolated in 2001 from children with respiratory-tract disease that was not attributable to other previously known respiratory viruses (van den Hoogen *et al.*, 2001). Although recently identified, the virus is thought to have circulated in the human population for at least 50 years (van den Hoogen *et al.*, 2004; Williams *et al.*, 2006) and infections have been reported globally (Boivin *et al.*, 2002; Esper *et al.*, 2003; Mao *et al.*, 2008; Nissen *et al.*, 2002; Pierangeli *et al.*, 2007; Stockton *et al.*, 2002). hMPV is a single-stranded, negative-sense RNA virus, classified in the family *Paramyxoviridae* alongside other mammalian respiratory pathogens, including human respiratory syncytial virus (hRSV) and human parainfluenza viruses. Clinically, hMPV infection produces disease symptoms similar to those observed for hRSV infection, ranging from mild respiratory illness to bronchiolitis and pneumonia, although hMPV disease is less rampant (van den Hoogen *et al.*, 2003, 2004). Young children, the elderly and immunocompromised individuals are particularly susceptible to hMPV-associated disease, emphasizing the need to understand the role of antiviral immunity in the control of infection. Several studies have indicated a strong association between hMPV disease and asthma in both children (Garcia-Garcia *et al.*, 2007; Peiris *et al.*, 2003; Tauro *et al.*, 2008) and adults (Williams *et al.*, 2005), implicating hMPV in ongoing or long-term adverse effects.

Clinical infection with hMPV and hRSV in humans occurs throughout life, despite the fact that most individuals sustain humoral immune responses to both hMPV (Ebihara *et al.*, 2004; van den Hoogen *et al.*, 2004) and hRSV (Baumeister *et al.*, 2003; Ward *et al.*, 1983). Whilst the cellular immune response following hRSV infection is well-understood in humans (Welliver, 2008) and in animal models (Hall *et al.*, 1986; Rutigliano *et al.*, 2005), in hMPV infection it is incompletely described. Similarities to hRSV suggest that $CD8^+$ T cells are likely to be necessary to resolve hMPV infection in humans (Hall *et al.*, 1986). A role for cytotoxic T lymphocytes (CTLs) in the control of hMPV infection is supported by *in vivo* mouse studies showing increased hMPV titres in T cell-depleted mice

†These authors contributed equally to this work.

(Alvarez *et al.*, 2004), and protection against infection by adoptive transfer of hMPV-specific CTLs (Melendi *et al.*, 2007) and hMPV-directed T-cell vaccines (Herd *et al.*, 2006). However, the onset of the CTL-mediated immune response and the induction of immunoregulatory cytokines following infection with hMPV require further study.

In the present study, we examined the induction of the CTL-mediated immune response and the induction of cytokines and chemokines in hMPV-infected mice, and compared these with the responses induced in hRSV-infected mice. We demonstrate that hMPV infection results in an accumulation of virus-specific cytotoxic and gamma interferon (IFN-γ)$^{+}$CD8^{+} effector T cells in the airways and lungs at 7 days post-infection (p.i.). This T-cell immunity was associated with increased expression of Th1-type [interleukin (IL)-12 and IFN-γ] and antiviral (IFN-β) cytokines and the chemokines Mip-1α (CCL3), Mip-1β (CCL4), Mig (CXCL9), IP-10 (CXCL10) and CX3CL1 (fractalkine). A moderate increase in the expression of Th2-type cytokines (IL-4 and IL-10) was also observed. No effector CTLs were detected in the regional lymph nodes or spleen at 7 days p.i., although a strong memory response could be recalled from the spleen at 21 days p.i. A similar pattern of cell-mediated responses was observed in hRSV-infected mice, highlighting the similarities in immunity to these respiratory pathogens. We discuss these findings in the context of other studies comparing hMPV and hRSV infection (Guerrero-Plata et al., 2005; Huck *et al.*, 2007). The findings support our previous observation that a CTL-epitope vaccine can protect against hMPV infection in mice (Herd *et al.*, 2006) and offer encouragement for the prospect of developing effective immunotherapies.

RESULTS

Virus infection of the lung

BALB/c mice were infected intranasally with 10^{5} $TCID_{50}$ of either hMPV or hRSV. At 1 and 2 days p.i., significant weight loss was evident in hMPV-infected mice, but not in hRSV-infected mice (Fig. 1a). However, no other signs of illness were detected in either group. At 5 days p.i., viral load was $10^{2.9}$ and $10^{3.2}$ $TCID_{50}$ per left lung for hMPV- and hRSV-infected mice, respectively (Fig. 1b). At 8 days p.i., viral load was below the detection limit of $10^{1.6}$ $TCID_{50}$ per left lung (data not shown). The lungs of infected mice showed pathology consistent with interstitial pneumonia (i.e. bronchial epithelial-cell damage and infiltration by inflammatory cells), with more severe pathology evident in hRSV-infected lungs (Fig. 1c). Cellular infiltration occurred predominantly around and within the alveoli and within the airways at approximately 5 days p.i. with either hMPV or hRSV.

Accumulation of virus-specific IFN-γ^{+}CD8^{+} T cells in airways and lungs

An important CTL-mediated effector mechanism during virus infection is secretion of antiviral cytokines such as IFN-γ. We evaluated *ex vivo* virus-specific CTL activity from mouse lungs after infection with either hMPV or hRSV. Cells from the airways (bronchoalveolar lavage; BAL), lung tissue, regional lymph nodes and spleen were tested at 7 days p.i. for the presence of CD8^{+} T cells that produced IFN-γ specifically in response to short-term stimulation with virus-specific peptide. For hMPV-infected mice, CD8^{+} T cells from the airways (14 %) and lungs (18 %), but not the lymph nodes or spleen, produced IFN-γ when stimulated with 'GYI' epitope peptide (insets, Fig. 2a). A similar pattern of CD8^{+} T cells was seen for hRSV-infected mice when stimulated with 'SYI' epitope peptide (insets, Fig. 2b).

hMPV-specific cytotoxic T cells in airways and lungs

Antiviral CTLs are important for resolution of infection with a number of viruses, including hRSV. To determine whether CTL activity was induced by hMPV infection and whether it was similar to that induced by hRSV infection, cells were collected from the airways (BAL), lungs, draining lymph nodes and spleen at 7 days p.i. and were tested in a mini-CTL assay. Airway and lung cells from hMPV-infected mice killed 'GYI'-target cells (14 and 7 % cytotoxicity at an effector-to-target cell ratio of 12 : 1, respectively) (Fig. 2a). Similarly, airway and lung cells from hRSV-infected mice killed 'SYI'-target cells (34 and 12 % cytotoxicity, respectively) (Fig. 2b). In contrast, cells from the regional lymph nodes and spleen of virus-infected mice failed to kill target cells. The presence of detectable CTL activity correlated with the induction of IFN-γ^{+}CD8^{+} T cells (insets, Fig. 2).

To investigate whether virus-specific memory CTLs were present, cells were collected from the spleen at 21 days p.i., restimulated *in vitro* with virus-specific peptide for 6 days and then tested in a mini-CTL assay. Restimulated spleen cells from hMPV-infected mice and from hRSV-infected mice showed substantial killing of peptide-labelled target cells (40–60 %), even at the lowest effector-to-target cell ratio of 2 : 1 (Fig. 3).

In summary, pulmonary infection with hMPV induces an effector CTL response detectable *ex vivo* in cells from the airways and lungs, but not from regional lymph nodes or spleen, at 7 days p.i. The response is similar to, but slightly lower than, that seen for hRSV infection. The data also indicate that a memory CTL response specific for the CTL epitopes in the M2 proteins could be recalled at 21 days p.i.

hMPV infection modifies the expression of cytokines and chemokines in lungs

The production of cytokines and chemokines plays a vital role in mediating recovery from virus infections (reviewed by Ramshaw *et al.*, 1997). It was thus relevant to ask whether antiviral and/or immunoregulatory cytokine and chemokine expression was altered following hMPV

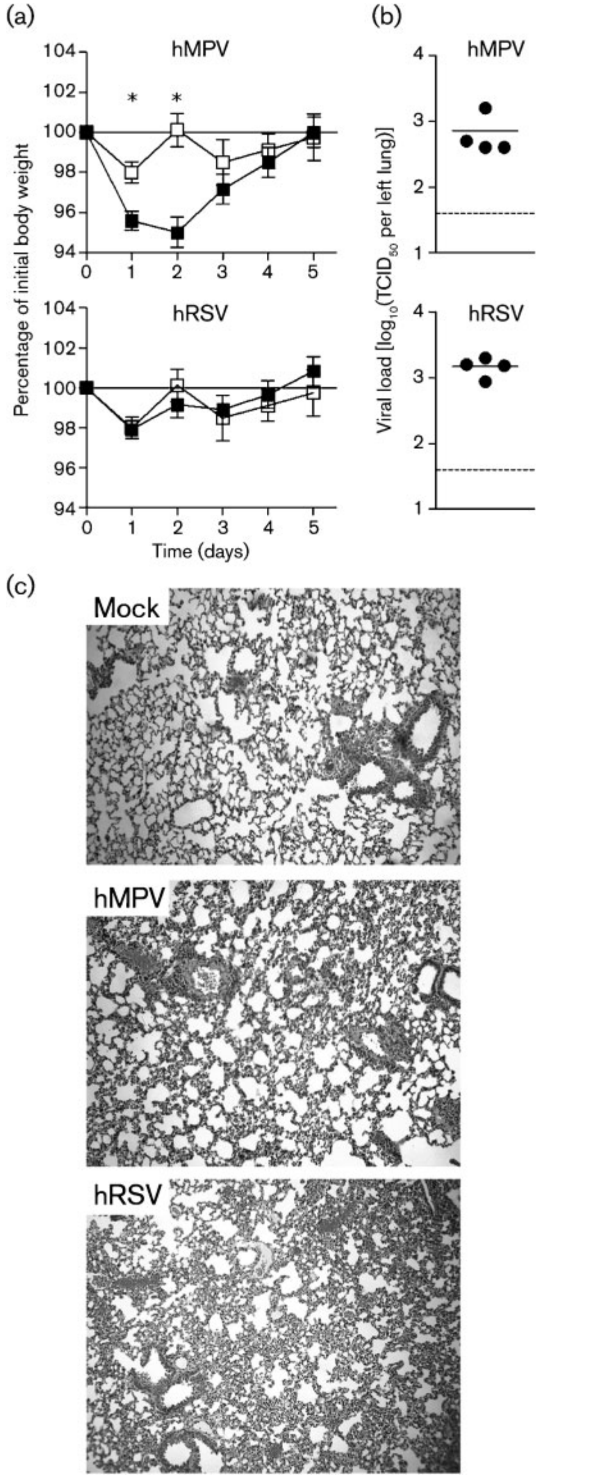

Fig. 1. hMPV and hRSV infection. BALB/c mice (n=4 per group) were infected intranasally with 10^5 $TCID_{50}$ of either hMPV or hRSV (■) or were uninfected (□). (a) Weight was monitored daily, and results are shown as mean percentage weight change ± SEM (*$P<0.05$). (b) Viral load in lungs at 5 days p.i. was determined by immunofocus assay. Results are shown as $\log_{10}$($TCID_{50}$ per left lung). The solid line represents the geometric mean. (c) Histopathological changes in lungs at 5 days p.i. were examined after haematoxylin/eosin staining. Representative sections are shown for uninfected, hMPV- and hRSV-infected mice (magnification, ×10).

infection. RT-PCR analysis indicated that mRNAs encoding the Th1-type (IL-12) and Th2-type (IL-4 and IL-10) cytokines were expressed at >10-fold higher levels in the lungs of hMPV- and hRSV-infected mice compared with uninfected mice (Fig. 4). In addition, mRNAs encoding T-cell attractant and/or pro-inflammatory chemokines (Mip-1β, Mig and CX3CL1) were expressed at >30-fold higher levels in virus-infected mice compared with uninfected mice. ELISA analysis of lung homogenates showed that levels of the Th1-type (IFN-γ) and antiviral (IFN-β) cytokines, as well as chemokines (Mip-1α and IP-10), were enhanced in virus-infected mice compared with uninfected mice (Fig. 5).

In conclusion, these data suggest that hMPV infection creates a local milieu conducive to development of both innate and adaptive immune responses. Furthermore, within the context of the cytokines and chemokines assayed, the outcome of hMPV infection parallels that of hRSV infection.

DISCUSSION

Human metapneumovirus is an important respiratory pathogen causing infections worldwide, with symptoms similar to those seen in hRSV and parainfluenza virus infections (van den Hoogen *et al.*, 2004). The strong association between hMPV infection and asthma in both children (Garcia-Garcia *et al.*, 2007; Peiris *et al.*, 2003) and adults (Williams *et al.*, 2005), and the ability of hMPV infection to exacerbate hRSV disease (Bosis *et al.*, 2005; Semple *et al.*, 2005), illustrate the need to improve our understanding of hMPV-induced T-cell immunity, particularly as a prelude to therapeutic intervention. As hMPV can establish productive infection in the upper and lower respiratory tract (Alvarez *et al.*, 2004; Hamelin *et al.*, 2005; van den Hoogen *et al.*, 2001), hMPV-expressed proteins have the potential to function as targets of $CD8^+$ CTL responses. Similarities to other, more widely studied viruses, such as hRSV (Braciale, 2005), suggest that this process may be associated with reduced virus replication and the duration or intensity of infection.

In the present study, intranasal infection of mice with hMPV was used as a model for natural infection in humans. The kinetics of virus replication, the self-limiting infection and the early onset of inflammation are similar to the responses induced by hRSV infection and are in accordance with previous studies (Hamelin *et al.*, 2005; Huck *et al.*, 2007). Weight loss was more severe after hMPV infection, in accord with findings elsewhere (Huck *et al.*, 2007). Our observation that histopathology of lungs was more severe after hRSV infection than hMPV infection

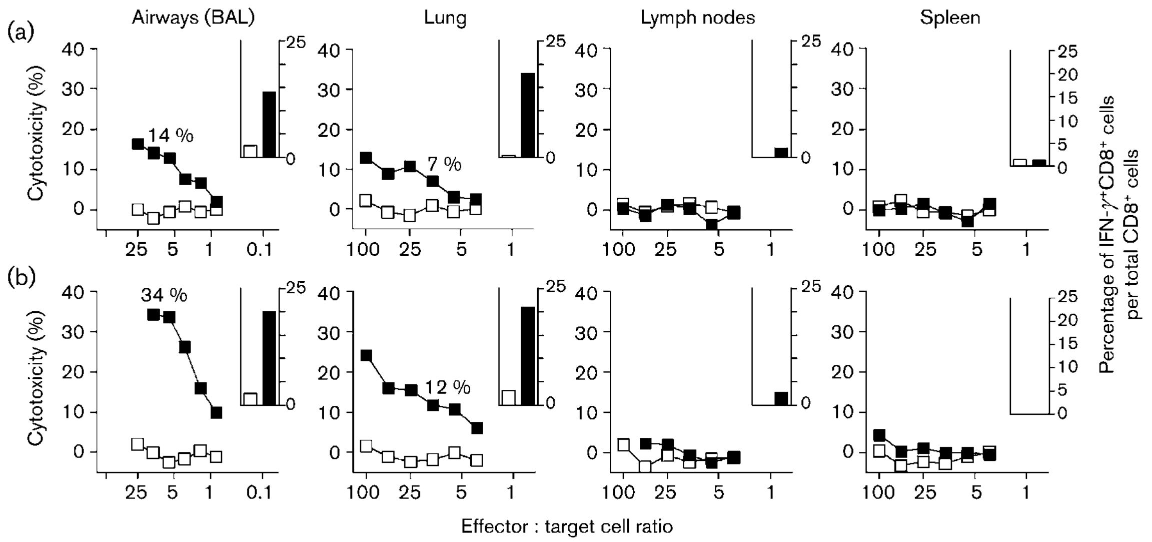

Fig. 2. hMPV and hRSV induce virus-specific cytotoxic and IFN-γ^+CD8$^+$ effector T cells in airways and lungs. Mice (n=4 per group) were infected intranasally with 10^5 $TCID_{50}$ of either hMPV (a) or hRSV (b). Cells from the airways (BAL), lungs, regional lymph nodes and spleen were collected at 7 days p.i., pooled per group and evaluated *ex vivo* for cytotoxicity against target cells with (■) or without (□) virus-specific M2 peptide. Results are expressed as percentage cytotoxicity. Numbers represent peptide-specific cytotoxity at an effector-to-target cell ratio of 12 : 1. Bar graphs (insets) represent IFN-γ^+CD8$^+$ T cells as a percentage of total CD8$^+$ T cells (from four mice per group, pooled) after 6 h stimulation with (filled bars) or without (empty bars) M2 peptide.

(Fig. 1c) is in contrast to that of Huck *et al.* (2007) and may reflect the virulence of hMPV, although both laboratories used primary hMPV strain A isolated from human patients. We further extend the findings on T-cell recruitment (Huck *et al.*, 2007) to show no major difference in accumulation of epitope-specific CTLs in the lung airways between hMPV and hRSV infection (Fig. 2). The CTL epitope ('GYI') in the M2-1 protein of hMPV occurs at a near-identical position to the CTL epitope ('SYI') in the M2 protein of hRSV, and was therefore chosen to monitor cell-mediated immunity. At 7 days p.i., accumulation of CD8$^+$ T cells capable of producing IFN-γ in response to stimulation with the 'GYI' epitope occurred in the airways and lungs. This was associated with CTL activity specific for target cells expressing the 'GYI' epitope. No such response could be detected in the regional lymph nodes or spleen at 7 days p.i., supporting the concept that a local response at the site of infection may be a first line of defence in CTL-mediated virus control during the early stages of infection. Although no virus-specific CTL activity was evident in the spleen at 7 days p.i., a strong response could be recalled at 21 days p.i. (Fig. 3), reflecting that seen in a previous study (Alvarez & Tripp, 2005). This suggests that a systemic spread of response from the lung and airways may be associated with the development of memory.

Our studies do not support the delay in the onset of CTL responses following murine infection reported previously (Alvarez *et al.*, 2004). This discrepancy may reflect that these authors examined splenic and not airway mononuclear cells. The CTL response that we observe following experimental infection was recorded 3 days earlier than reported by Melendi *et al.* (2007). Our findings are in accord with those showing that CD8$^+$ T cells are required for clearance of primary hMPV from the lung; for a number of weeks after primary infection, mice are protected by virtue of this CD8$^+$ T-cell response against further hMPV challenge (Kolli *et al.*, 2008). Studies elsewhere (Alvarez & Tripp, 2005; Kolli *et al.*, 2008) show that CD4$^+$ T cells, as well as CD8$^+$ T cells, play an antiviral role, and the two subsets act together synergistically to effect hMPV clearance from the lung. Depletion studies, however, indicate that protection from reinfection can be mediated by an intact CD8$^+$ T-cell compartment alone in the absence of CD4$^+$ T cells and of neutralizing antibodies (Kolli *et al.*, 2008). This is in contrast to hRSV primary infection of mice, in which absence of CD8$^+$ T cells provided a greater protective effect against disease than an absence of CD4$^+$ T cells (Graham *et al.*, 1991; Kolli *et al.*, 2008). Similarly, whilst CD8$^+$ T cells appear to be sufficient to control hMPV reinfection, in hRSV reinfection CD4$^+$ T cells (and antibody) are required (Kolli *et al.*, 2008).

Whilst CD4$^+$ and CD8$^+$ CTL responses serve to clear current infection and protect against future infection, they also contribute to clinical disease and lung pathology, although the pathophysiology is not well-understood (Kolli *et al.*, 2008). The effects are less severe if CD4$^+$ rather than

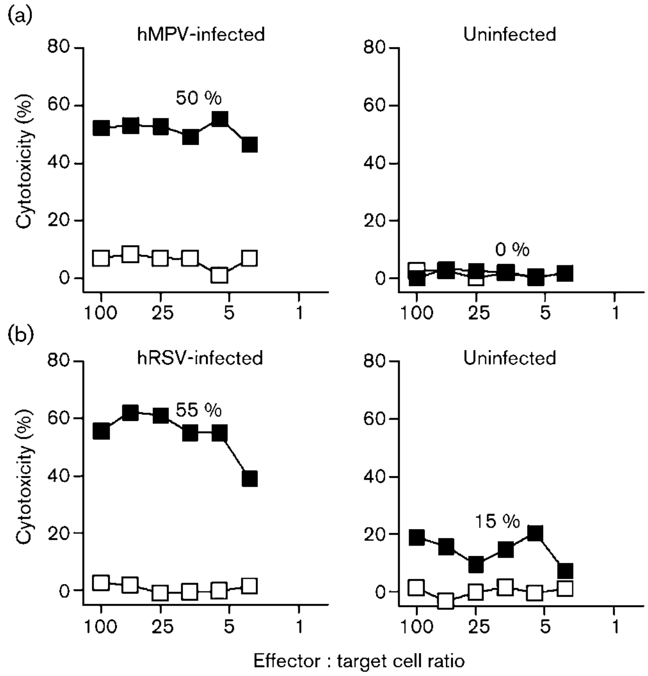

Fig. 3. hMPV and hRSV induce virus-specific memory CTLs in the spleen at 21 days p.i. Mice (n=4 per group) were infected intranasally with 10^5 $TCID_{50}$ of either hMPV (a) or hRSV (b) or were uninfected. Spleen cells were harvested at 21 days p.i., pooled per group and restimulated *in vitro* with virus-specific M2 peptide for 6 days before being evaluated for cytotoxicity against target cells with (■) or without (□) the appropriate peptide. Results are expressed as percentage cytotoxicity. Numbers represent peptide-specific cytotoxity at an effector-to-target cell ratio of 12 : 1.

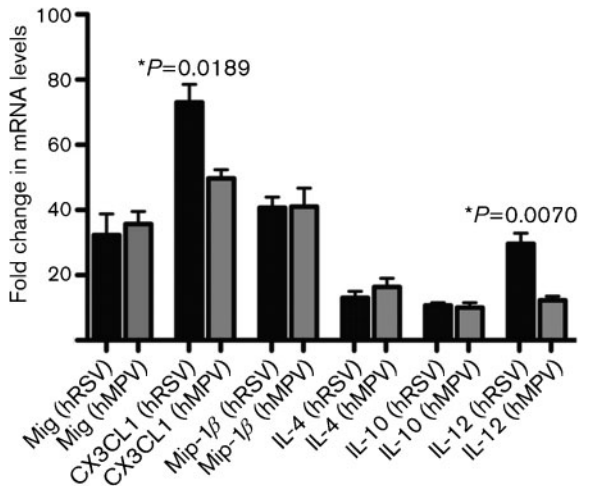

Fig. 4. Immunoregulatory cytokine and chemokine expression is upregulated in lungs. Mice (n=3 per group) were infected intranasally with $10^{5.7}$ p.f.u. of either hMPV (shaded bars) or hRSV (filled bars) or were uninfected. Cytokine and chemokine expression in the lungs at 7 days p.i. was quantified by real-time PCR. Results are expressed as fold change over mRNA levels of these cytokines and chemokines in the lungs of uninfected mice (mean ± SEM). Cytokines are interleukins and chemokines are Mip-1β, Mig and CX3CL1.

$CD8^+$ cells are depleted (Alvarez *et al.*, 2004; Kolli *et al.*, 2008) and a recent paper reported that hMPV infection was more severe in aged mice, a finding that corresponded to, among other things, an increase in $CD4^+$ T cells being recruited to the respiratory tract (Darniot *et al.*, 2009). This suggests that $CD8^+$ cells are not primarily responsible for the detrimental effects of immune-response induction following natural infection with hMPV, and underscores a putative advantage for a Th1 bias in the cellular immune response to hMPV infection.

In this study, hMPV infection was seen to enhance the expression of Th1-type (IL-12, IFN-γ) and antiviral (IFN-β) cytokines. Most laboratories agree on the upregulation of IFN-γ after hMPV infection (Guerrero-Plata *et al.*, 2005; Hamelin *et al.*, 2005; Huck *et al.*, 2007). In the current study, the local accumulation of $CD8^+$ IFN-γ-producing cells correlated with the hMPV-directed cytolytic activity of mononuclear cells at these sites (Fig. 2). Additionally, IFN-γ coordinates a diverse array of cellular programmes that will impact upon virus-infected cells through transcriptional regulation of immunologically relevant genes.

Only moderate (Fig. 4) or no (Guerrero-Plata *et al.*, 2005; Huck *et al.*, 2007) upregulation of Th2-type cytokine IL-10 was recorded following infection, consistent with the interpretation of a Th1-biased response. In contrast, substantially augmented IL-10 expression reported by Alvarez & Tripp (2005) at 7–28 days suggests induction of a Th2-type response in the later stages of infection in their model. This latter result is in accord with Barends *et al.* (2002), who demonstrated that some paramyxoviruses, such as hRSV, enhance a Th2 response.

We found that hMPV and hRSV had similar potency in induction of pro-inflammatory cytokines in the context of those cytokines that we studied and at the time point that we examined (7 days p.i.). Guerrero-Plata *et al.* (2005) showed that, whilst hRSV and hMPV infections induced similar production of some cytokines, a further set of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) were induced differentially. In accord with findings elsewhere (Douville *et al.*, 2006), they concluded that hMPV induces lower levels of canonical inflammatory cytokines than hRSV, although hMPV was a more potent inducer of others, e.g. granulocyte–macrophage colony-stimulating factor, IFN-γ and IFN-α. We show enhanced expression of several chemokines (Mip-1α, Mip-1β, Mig, IP-10 and CX3CL1) following hMPV infection (Figs 4 and 5). Upregulation of Mip-1α (CCL3) was also recorded by Hamelin *et al.* (2005). Notably, peak levels of Mip-1α in their study occurred at the height of virus infection (5–7 days p.i.). These results are in contrast to those of Guerrero-Plata *et al.* (2005), who did not record the production of Mip-1α following hMPV infection.

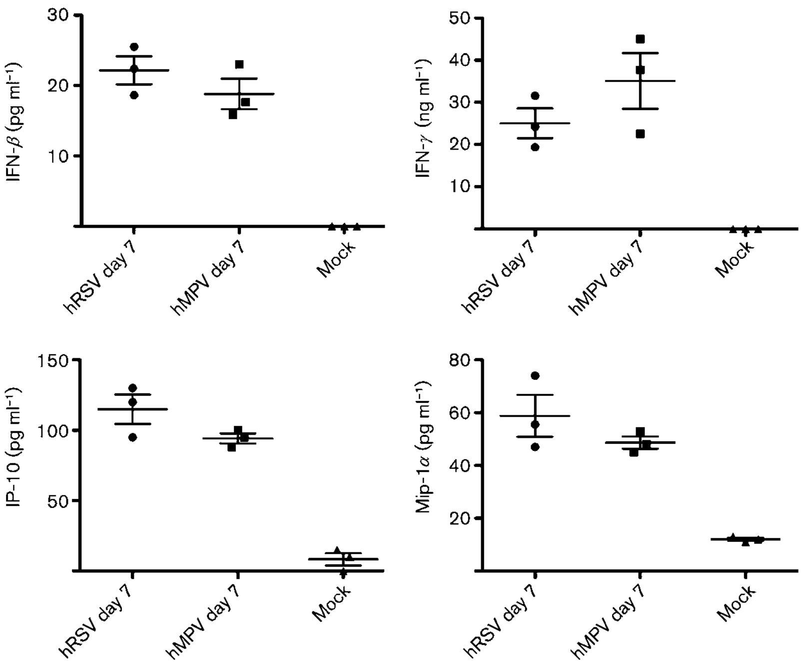

Fig. 5. Immunoregulatory cytokines are upregulated in lungs. Mice (n=3 per group) were infected intranasally with $10^{5.7}$ p.f.u. of either hMPV or hRSV or were uninfected (mock). Cytokine concentration in lung homogenates at 7 days p.i. was quantified by ELISA. Results are shown as data points for individual mice, mean ± SEM. Cytokines are interferons and chemokines are Mip-1α and IP-10.

The discrepancies noted above in cytokine/chemokine production following hMPV infection may reflect the isolate of virus (laboratory-adapted versus primary isolate) used in the various studies, differences in the cytokines examined, the time points examined and methodological read-outs (mRNA expression/protein concentration/intracellular cytokine staining).

Chemokines are likely to play a role in regulating the immune response by induction of local inflammation and as chemoattractants for mononuclear cells. Both of these effects predispose to effective innate and adaptive immune responses. Such responses may play a significant role in the clinical manifestations of hMPV and hRSV infection observed in humans, and contribute to the less severe disease induced by hMPV (Williams *et al.*, 2006).

In this study, M2 CTL epitopes were used to evaluate cell-mediated immunity, as they occur at a near-identical location and with identical major histocompatibility complex (MHC) class I anchor residues (i.e. MHC restriction) in both hMPV and hRSV. We have previously reported other similarities in immunogenic regions and CTL-epitope sequences between hMPV and hRSV (Herd *et al.*, 2008), probably reflecting the taxonomic and pathogenic relatedness of the two viruses.

Studies from our laboratory and others indicate that a repertoire of hMPV-specific CTL responses is engendered to a range of epitopes in humans (Herd *et al.*, 2008) and mice (Herd *et al.*, 2006; Melendi *et al.*, 2007). In humans, the amino acid sequences of the identified CTL epitopes are conserved amongst many, and in some cases all, identified hMPV genotypes and strains (Herd *et al.*, 2008), suggesting that natural infection with a given strain might engender cross-protection against others. A broad-repertoire response is likely to protect against the emergence of putative escape mutants containing mutations in individual epitopes. In humans, we have shown that an hMPV-directed virus-specific memory response appears to persist for at least several years following clinical hMPV infection (Herd *et al.*, 2008) [although it cannot be ruled out that this may be due to subsequent subclinical infection(s) reinforcing memory]. Nonetheless, it is clear that, in humans, natural infection generates an IFN-γ-secreting CTL response. Whilst recall of this response does not seem to prevent reinfection (as evidenced by recurrent bouts of clinical infection throughout life), it may serve to halt serious lower respiratory-tract disease in healthy adults. This may not be the case in the very young, the elderly or immunocompromised individuals.

In conclusion, we demonstrate a local T-cell response in the lungs and airways of mice infected with the recently identified respiratory pathogen hMPV. Along with our earlier data reporting memory CTL responses in humans (Herd *et al.*, 2008), these results suggest that hMPV-mediated CTL and cytokine/chemokine responses engendered as a result of infection might contribute to control of the virus, and provide a rationale for immunointervention. Our results also demonstrate similarities in cellular immune-response induction by infection with hMPV and hRSV, a phylogenetically related virus with similar biological and clinical features.

METHODS

Cell lines and epitope peptides. LLC-MK2 or HEp-2 cells (ATCC) were used for virus production and quantification. Cell lines were maintained in Opti-MEM (Invitrogen) or Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 5 % fetal bovine serum (FBS). P815 cells (H-2^d, mastocytoma) (Sir Albert Sakzewski Virus Research Centre, Australia) were used as targets in cytotoxicity assays and were maintained in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 IU penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 10 % FBS. The CTL epitopes 'GYI' and 'SYI' are both H2-K^d-restricted and occur at similar locations in the M2 proteins of hMPV and hRSV, respectively (Table 1). These epitopes were synthesized as peptides (>85 % purity; Mimotopes), dissolved at 10 mg ml^{-1} in DMSO and diluted into assays as required.

Virus preparation and quantification. hMPV [lineage A: AUS-001; CAN97-83 (Hamelin *et al.*, 2005)] was prepared in LLC-MK2 cells and hRSV was prepared in HEp-2 cells, as described previously (Alvarez *et al.*, 2004; Herd *et al.*, 2006; Woo *et al.*, 2006). In brief, virus was prepared by infecting cells in 225 cm^2 flasks at an m.o.i. of 0.1. After adsorption for 2 h at 37 °C, the inoculum was removed and the cells were washed before addition of fresh medium (40 ml). For hMPV, trypsin (Invitrogen) was added during infection (5 μg ml^{-1}) and every other day (2.5 μg ml^{-1}). Cultures were incubated until 70–90 % cytopathic effect was apparent, usually within 7 days. Virus was obtained from the culture supernatant and by freeze–thawing the cells. If necessary, virus was concentrated by ultrafiltration using Centricon Plus-20 filter units (Millipore; 100 K nominal molecular mass limit). Both viruses were quantified by immunofocus assay as described previously (Herd *et al.*, 2006; Woo *et al.*, 2006). In brief, subconfluent cell monolayers in a 96-well plate were infected with 100 μl of serial dilutions (10-fold for virus, 4-fold for lungs) and incubated for 2 h at 37 °C, before addition of 100 μl fresh medium with 10 % FBS. For hMPV, trypsin was added during infection and then every other day. After 6 days, infected wells were identified by using virus-specific antibody (anti-N monoclonal for hMPV or polyclonal antibody for hRSV; Chemicon) followed by species-appropriate developing reagents (anti-mouse or anti-goat, horseradish peroxidase-labelled) and DAB substrate with metal enhancer (Sigma). Results were expressed as $TCID_{50}$ values. For lung samples, the lower limit of detection was $10^{1.6}$ $TCID_{50}$ per left lung.

Infection and sampling of mice. BALB/c mice (H-2^d, female, approx. 20 g) were supplied by the Animal Resources Centre (Perth, Australia) and maintained under specific-pathogen-free conditions. Mice were anaesthetized with ketamine/xylazine prior to intranasal administration of virus (10^5 $TCID_{50}$ in 60 μl) or Hanks' balanced salt solution (60 μl) as a control. Clinical illness was monitored and body weight was recorded daily. At 5 days p.i., lungs were removed then snap-frozen and stored at −70 °C before being homogenized for determination of viral load. At 7 days p.i., lungs were collected for histopathology and also for determination of cytokine/chemokine levels.

Histopathology staining. Histopathological examination was performed on lung samples isolated from hMPV-infected mice as described by Hertz *et al.* (2001). Briefly, lung tissue representing the central (bronchi–bronchiole) and peripheral (alveoli) airways was fixed in 10 % phosphate-buffered formalin, sectioned and stained with haematoxylin and eosin.

Preparation of effector cells. At 7 days p.i., effector cells were obtained from the airways (by BAL), whole lung tissue, regional lymph nodes (mediastinal and tracheobronchial) and spleen for determination of virus-specific CTL activity. Briefly, mice were euthanized with ketamine/xylazine and then exsanguinated via the abdominal aorta. For BAL, the thorax was opened, the diaphragm pierced, the anterior ribcage removed and a small incision made between the cartilage rings of the trachea. A 20G luer stub adaptor needle was inserted and BAL was performed with 3 vols (0.8 ml each) of Hanks' balanced salt solution. For isolation of cells from parenchyma, lungs were cut into 1 × 1 mm pieces and treated with collagenase A (Roche; 4 mg per 2 ml per lung sample, 37 °C for 1 h with agitation). Single-cell suspensions were then prepared from the lungs, lymph nodes and spleen and by pressing through cell strainers (100 μm) with a syringe plunger. At 21 days p.i., spleen cells were obtained and restimulated *in vitro* with virus-specific peptide for 6 days.

Cytotoxic T-cell and intracellular cytokine mini-assays. Cytotoxic T-cell activity was measured in a ^{51}Cr-release assay modified for small cell numbers, as described elsewhere (Ostler *et al.*, 2001). Briefly, effector cells (starting at 2×10^5, or 5×10^4 for airway samples) were incubated with ^{51}Cr-labelled target cells (2×10^3), with or without peptide (10 μg ml^{-1}), in a 96 'V'-well plate in a total volume of 100 μl. After 6 h at 37 °C, 25 μl cell-free supernatant was collected and radioactivity was determined. Peptide-specific cytotoxicity was defined as (percentage cytotoxicity for targets with peptide)−(percentage cytotoxicity for targets without peptide). For intracellular cytokine analysis, cells (2×10^5 or 5×10^4) were cultured for 6 h in a 96 'U'-well plate, with or without 10 μg peptide ml^{-1} in a volume of 0.2 ml. Brefeldin A (GolgiPlug; PharMingen) was added at 1 μl ml^{-1} for the last 4 h to facilitate intracellular accumulation of cytokine. Cells were washed and surface-stained with anti-CD8 [53-6.7, phycoerythrin (PE)/Cy5-labelled] or an isotype control. Intracellular cytokine staining was performed with anti-IFN-γ (XMG1.2, PE-labelled) or an isotype control, using a CytoFix/CytoPerm kit (PharMingen) according to the manufacturer's instructions. Cells were analysed on a Quanta SC flow cytometer (Beckman Coulter).

Quantification of chemokine and cytokine mRNA expression in lungs. Total RNA was isolated by using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions, after which 5 μg total RNA was reverse-transcribed using an oligo(dT) primer and 260 U reverse transcriptase μl^{-1} (Promega), according to the manufacturer's instructions. Real-time PCR was performed on a

Table 1. CTL epitopes at similar locations in the M2 proteins of hMPV and hRSV

Virus	Protein	Position*	CTL epitope† (abbreviation)	MHC class I restriction	Reference
hMPV	M2-1	81–89	G**Y**IDDNQS**I** ('GYI')	H-2K^d	Baumeister *et al.* (2003)
hRSV	M2	82–90	S**Y**IGSINN**I** ('SYI')	H-2K^d	Openshaw *et al.* (1990)

*Position is specified as the amino acid numbers of the M2-1 genes of hMPV (GenBank accession no. ACJ70116) and hRSV (GenBank accession no. Q14974).

†MHC anchor residues are shown in bold.

Rotor-Gene RG-3000 (Corbett Research), using Quantitect Primer Assay kits (Qiagen), based on quantification of the SYBR green I fluorescent dye. Specificity of the amplification was evaluated by a melting-curve analysis of PCR products. Real-time PCR results were expressed as 'fold change in mRNA expression', comparing infected samples with the experimental controls (mock-inoculated + vehicle). The Relative Expression Software Tool (REST) was used to calculate differences in mRNA levels, which were first normalized to levels of the housekeeping gene hypoxanthine–guanine phosphoribosyltransferase (HPRT).

ELISAs for mouse cytokines. The concentration of Mip-1α, IFN-α, IP-10 and IFN-γ in samples was determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical analysis. Data are presented as means ± SEM. Mean values were compared by Student's *t*-test and $P<0.05$ was considered significant.

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